Sea Anemone Actinoporins: The Transition from a Folded Soluble State to a Functionally Active Membrane-Bound Oligomeric Pore

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Abstract: Actinoporins are a family of 20-kDa, basic proteins isolated from sea anemones, whose activity is inhibited by preincubation with sphingomyelin. They are produced in monomeric soluble form but, when binding to the plasma membrane, they oligomerize to produce functional pores which result in cell lysis. Equinatoxin II (EqtII) from *Actinia equina* and Sticholysin II (StnII) from *Stichodactyla helianthus* are the actinoporins that have been studied in more detail. Both proteins display a β-sandwich fold composed of 10 β-strands flanked on each side by two short α-helices. Two-dimensional crystallization on lipid monolayers has allowed the determination of low-resolution models of tetrameric structures distinct from the pore. However, the actual structure of the pore is not known yet. Wild-type EqtII and StnII, as well as a nice collection of natural and artificially made variants of both proteins, have been produced in *Escherichia coli* and purified. Their characterization has allowed the proposal of a model for the mechanism of pore formation. Four regions of the actinoporins structure seem to play an important role. First, a phosphocholine-binding site and a cluster of exposed aromatic residues, together with a basic region, would be involved in the initial interaction with the membrane, whereas the amphipathic N-terminal region would be essential for oligomerization and pore formation. Accordingly, the model states that pore formation would proceed in at least four steps: Monomer binding to the membrane interface, assembly of four monomers, and at least two distinct conformational changes driving to the final formation of the functional pore.

Keywords: Actinoporins, equinatoxin, sticholysin, pore-forming toxin, lipid-protein interaction, membrane, pore, oligomerization.

INTRODUCTION

Most of the proteins in Nature have evolved to exist either in aqueous solution or integrated in lipid membranes. Pore-forming toxins (PFTs) are a paradigmatic exception to that rule [1]. These proteins are synthesized in a water-soluble form with the fascinating ability to interact with membranes and build a pore after changing their conformation. Thus, having developed their functionality, PFTs behave as integral membrane proteins. Despite the intrinsic interest of PFTs as virulence factors in a number of pathologies [2,3], PFTs are primarily being employed as excellent model systems to address different problems of modern Biology. In particular, PFTs show that the same primary structure can fold into two different structures, i.e., that the environment influences the energy landscape of a protein [4]. The knowledge of the mechanism by which PFTs insert in the membrane, as well as the energetics of the process, may also be of great help in the solution of the so-called membrane protein folding problem [5,6]. In addition, the recent findings that lipid-protein interactions may play a significant role in the misfolding and aggregation of proteins [7] open the possibility of considering PFTs also as good models for the study of proteins responsible for the devastating "conformational diseases" [8].

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PFTs are usually classified regarding the structural motif that forms the pore walls. Then, α-PFTs insert α-helices in the membrane, whereas β-PFTs use β-barrels to span the membrane [9]. Actinoporins are a family of eukaryotic α-PFTs produced by sea anemones [10-12]. In contrast to the majority of α-PFTs, actinoporins are small proteins (~20 kDa) with only one domain. This review summarizes the extensive research made on actinoporins during the last years and finishes with a perspective on what should be done in the future in order to better understand the functionality of this family of proteins.

BIOLOGICAL SOURCE AND HETEROLOGOUS EXPRESSION

Actinoporins, together with many other toxic proteins and peptides, are commonly thought to be produced by sea anemones inside the nematocysts, stinging organelles present in the coelenterates (Cnidaria), though other locations cannot be ruled out [12]. Having this localization, actinoporins are believed to participate in functions like predation, defense, and digestion [13]. At the present time, 25 sea anemone species have been described to produce actinoporin-like activities [12,14-16]. A detailed list of the actinoporins that have been isolated so far is presented in Table 1. As can be seen, actinoporins are characterized by a molecular weight (MW) of ~ 20 kDa. Their isoelectric point (pI) is generally basic, and usually above nine. Only epiactin-A (pI = 6.4) and especially Src-I (pI = 4.8) have been described as acidic acti-

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Table 1. Molecular Features of the Actinoporins Whose Isolation has been Reported*

Species	Toxins	MW (kDa)	pI	Inhibition	Reference
Actineria villosa	Avt-I	19	9.2	SM	[14]
Actinia cari	Caritoxin I	19.8	9.4	SM	[88]
	Caritoxin II	19.8	10	SM	
Actinia equina	Equinatoxin I	19	9.8	SM	[19]
	Equinatoxin II	19	10.5	SM	
	Equinatoxin III	19	10.5	SM	
Actinia tenebrosa	Tenebrosin A	19.8	> 9.4	n.d.	[89,90]
	Tenebrosin B	19.5	> 9.4	n.d.	
	Tenebrosin C	19.8	> 9.4	SM	
Anthopleura japonica	"Hemolysin I"	19.5	> 9	SM	[91]
	"Hemolysin II"	19	8	n.d.	
Bunodosoma caissarum	Caissarolysin I	19.8	> 9	SM	[92]
Condylactis gigantea	C. g. toxin	18.3	8.9	SM	[93]
Entacmea quadricolor (Parasicyonis actinos-toloides)	Parasitoxin	18	7.9	n.d.	[94]
Epiactis prolifera	Epiactin A	19.5	6.4	n.d.	[95]
	Epiactin B	19.5	8.3	SM	
	Epiactin C	19.5	7.6	n.d.	
Heteractis magnifica	Magnificalysin I	19	9.4	n.d.	[96]
	Magnificalysin II	19	10	n.d.	
	Magnificalysin III	19	9.1	n.d.	
Phyllodiscus semoni	PsTX-20A	20	9.4	n.d.	[16]
Phymactis clematis	Coelenterolysin	n.d.	n.d.	SM	[32]
Pseudactinia varia	Variolysin	19.5	9.8	SM	[97]
Radianthus macrodactylus	RTX-A	20	9.8	SM	[98,99]
	RTX-S	20	9.8	SM	
	RTX-S II	19.3	10	SM	
	RTX-G	20	10.5	SM	
Sagartia rosea	Src-I	19.6	4.8	SM	[15,100]
Stichodactyla helianthus	Sticholysin I	19.4	9.2	SM	[20]
	Sticholysin II	19.3	9.8	SM	
Stichodactyla kenti	Kentin	18.0	9.2	SM	[101]
Stichodactyla mertensii	SmT-I	n.d.	n.d.	SM	[82]
	SmT-II	n.d.	n.d.	SM	

n.d. Not determined

*Apart from the references cited in the table, readers are referred to [11].

noporins. Another essential property that actinoporins have in common is their inhibition by preincubation with sphingomyelin (SM), which led to the proposal that this lipid was their membrane target [17]. Within this idea, two 10 kDa toxins from *Gyrostoma helianthus* and *Radianthus koseirensis* have been sometimes classified as actinoporins because they are inhibited by SM [18]. However, this inclusion is rather controversial due to their much smaller size, and should wait for a better inspection of their molecular properties.

The protocols for purification of actinoporins usually take advantage of their high isoelectric point by including a chromatographic step in carboxymethylcellulose [19,20]. Purification to homogeneity can be achieved by an additional size-exclusion chromatography. However, sea anemones often produce several isoforms of actinoporins, with only slight differences in terms of molecular weight or pI (Table **1**). For example, the actinoporins from *Actinia equina* have been shown to belong to a multigene family [21]. Thus, the isolation of pure molecular entities from the anemone is not an easy task. From this point of view, the heterologous production of actinoporins has long been sought, with the additional outlook of opening the possibility of obtaining mutant variants. Only six actinoporins have been produced as recombinant proteins (Table **2**) so far, all of them by means of *E. coli* IPTG-inducible systems. Purification strategies are similar to those employed for the wild-type proteins, except for a Ni-NTA chromatography when His-tagged proteins are expressed. As can be seen in Table **2**, the recovery yield varies depending on the actinoporin and on the inclusion of fusion partners. Very recently, it was proposed that the mRNA coding for StnI and StnII, two actinoporin isoforms from *Stichodactyla helianthus*, may form secondary structures blocking the ribosome-binding site and/or the initiation codon, thus reducing protein production in *E. coli* expression systems. The introduction of convenient silent mutations in the cDNA of StnI and StnII or their production as fusion proteins therefore resulted in a considerable increase of protein expression [22] (Table **2**).

MOLECULAR PROPERTIES AND ACTIVITY

The alignment of all reported actinoporins' sequences appears in Fig. (**1**). High degree of sequence identity (60 – 85%) and similarity (70 – 95%) are found among all of them, what would correlate with their similar sizes and pI. Lack of cysteine residues is observed in all the sequences, which constitutes another characteristic of the actinoporins' family. The most studied actinoporins are equinatoxin II (EqtII) from *Actinia equina* and those from *S. helianthus*, mainly sticholysin II (StnII). These proteins were first described as potent cytolytic and hemolytic proteins which were inhibited by preincubation with SM [17,23]. Further studies revealed that membrane damage due to transmembrane pore formation was responsible for the observed effects [24-26]. Now, it is generally accepted that all the actinoporins exert their toxicity by forming oligomeric cation-selective pores within membranes, which result in a colloid-osmotic shock that leads to cell death [27-29].

Both EqtII and StnII are able to form pores within model membranes in the absence of other proteins, which shows that no protein receptor is needed for membrane recognition [25,27,29,30]. In fact, taking into consideration the inhibition exerted by preincubation with SM, this lipid was proposed to be the membrane receptor for actinoporins [23]. Thus, the presence of SM in the target membrane would promote a higher degree of toxin binding that would also correlate with a higher rate of membrane permeabilization [28]. It was also

Table 2. Heterologous Systems for the Production of Actinoporins in *E. coli*

Protein	Plasmid	N-terminal extension	Yield (mg/liter of broth)	Reference
Equinatoxin II	pT7-7	No	1	[102]
	pET8c	TolE protein, His-Tag	11	[103]
Sticholysin I	pQE60	No	0.7	[22]
	pQE30	His-Tag	15	
	pQE60*	No	12.8	
Sticholysin II	pQE60	No	n.d.	[22,104]
	pQE30	His-Tag	5.0	
	pQE60*	No	5.2	
HmgIII	pQE30	His-Tag	3	[96]
SrcI	pBV220	No	15 - 20	[100]
	pTRX	Thioredoxin	20	[15]
Avt-I	pET Duet-1	No	1.9	[105]

* The cDNAs of StnI and StnII were cloned in pQE60 after introducing silent mutations within their 5' ends [22].
n.d. No expression was detected.

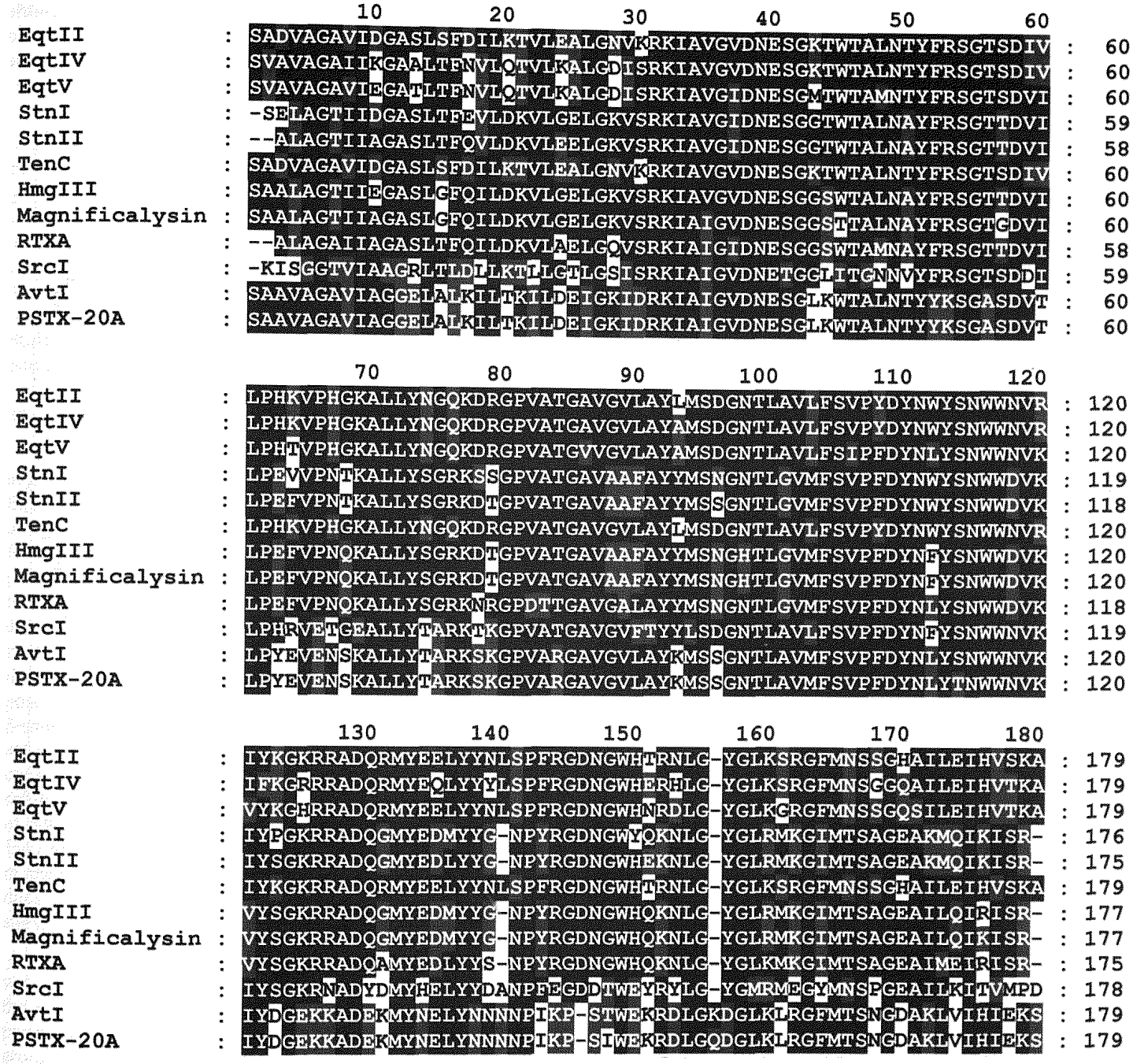

Fig. (1). Sequence alignment of actinoporins. CLUSTALW [81] was employed to perform the alignment. Figure was prepared with GeneDoc (http://www.psc.edu/biomed/genedoc). SwissProt accession numbers are: P61914 (EqtII), Q9Y1U9 (EqtIV), Q93109 (EqtV), P81662 (StnI), P07845 (StnII), P61915 (tenebrosin C, TenC), Q9U6X1 (magnificalysin III, HmgIII), P58691 (RTXA), Q86FQ0 (SrcI), Q5R231 (AvtI), Q8IAE2 (PsTX-20A). The sequence for magnificalysin was obtained from the bibliography [82]. It must be noted that EqtII and TenC have the same sequence. Black, grey, or white boxes indicate the different degree of conservation, in descending order, along all the reported actinoporins' sequences.

demonstrated by NMR that, in the presence of SM, EqtII promotes the formation of an isotropic lipid phase and slows down lipid mobility [31]. Interestingly, sea anemones have a phosphone analogue of SM, instead of SM, in their membranes. This analogue is unable to inhibit hemolysis nor binding to the actinoporin coelenterolysin [32]. However, the interaction of actinoporins with membranes seems to be much more complicated than a simple recognition of SM, as some model membranes without SM (e.g. mixtures of phosphatydilcholine (PC) and cholesterol (Ch) are also permeabilized by actinoporins [26,30]. In addition, unilamellar membranes composed only of SM are not permeabilized by StnII [30]. Furthermore, the association of EqtII to PC vesicles has also been reported [33]. All the studies carried out so far point out towards the idea that heterogeneous membranes are the best targets for actinoporins, an observation that has been related to the formation of lipid domains [30]. With this regard, it has been also shown that lipid-phase coexistence favours insertion of StnII and EqtII in model membranes [34,35] and pore formation by StnII in mammalian cells [36]. Taken together, all these facts suggest that most probably the rheologic and physicochemical properties of the

membrane are more important determinants for actinoporins correct functionality than its actual composition. In good agreement with this idea, actinoporins display temperature-dependent activity within a temperature range at which no significant changes on protein conformation would occur [34,37]. With this perspective, SM might be better considered as a very appropriate inductor of the optimal membrane properties for actinoporins' functionality than a real membrane receptor.

Recently, it has been observed that the inclusion of small quantities of anionic lipids renders membranes more sensitive to actinoporin-induced permeabilization. This would agree with the formation of a toroidal lipid pore [37]. Such a hypothesis is also sustained by ^{31}P NMR and Fourier-transform infrared spectroscopy (FTIR) experiments, which probe that EqtII induces non-lamellar lipid structures [38]. In addition, electron paramagnetic resonance (EPR) measurements show that there is no lipid-protein interaction at the lipid terminal methyl group, again suggesting the formation of a toroidal lipid pore [39]. The toroidal pore model was first proposed for magainin peptide [40,41]. In this model, the lipid bilayer bends back on itself, resulting in a conductive channel that is constructed by both the peptide and the head groups of the lipids.

In short, the functionality of actinoporins is directly related to their ability to establish strong interactions with lipid membranes. This sort of interaction is usually sustained by stretches of hydrophobic amino acids, which may eventually be visualized by means of a hydropathy plot. In fact, when applied to StnII and EqtII sequences (Fig. (**2A** and **B**)), three regions of high hydropathy values appear at first glance. As expected, they correspond to sequence stretches with a high content of hydrophobic amino acids. Interestingly, one of them is the N-terminus, a region that is also the most variable one within the different known actinoporins (Fig. (**2C**)). These facts would be enough by themselves to turn the spotlight on this N-terminus of actinoporins as a key region for their functionality. In the next sections, it will be shown how this suspicion seems to be well sustained by additional experimental data.

STRUCTURAL CHANGES ASSOCIATED WITH PORE FORMATION

The mechanisms of pore formation by PFTs are only partially understood [1]. It is generally accepted that these proteins initially bind to the target membrane as monomers guided by their affinity to some membrane component. Such an interaction results in an increase of toxin concentration at the lipid-water interface that leads to its oligomerization on the membrane surface. A non-conductive oligomer is therefore formed, which is often referred as "prepore". From this state, membrane permeabilization is achieved by substantial conformational changes within the toxin, which expose the hydrophobic patches of amino acids required for membrane insertion. Then, it seems clear that any particular PFT should be able to interact with membranes and oligomerize.

As mentioned above, actinoporins are able to interact with a variety of natural and model membranes. It has been hypothesized that such lipid-protein interactions may be related to the ability of the protein to adopt molten-globule states [42]. Remarkably, partially folded states that resemble molten-globules have been detected for EqtII [43,44] and StnII [45] and, under some experimental conditions, those partially folded states irreversibly aggregate [45,46].

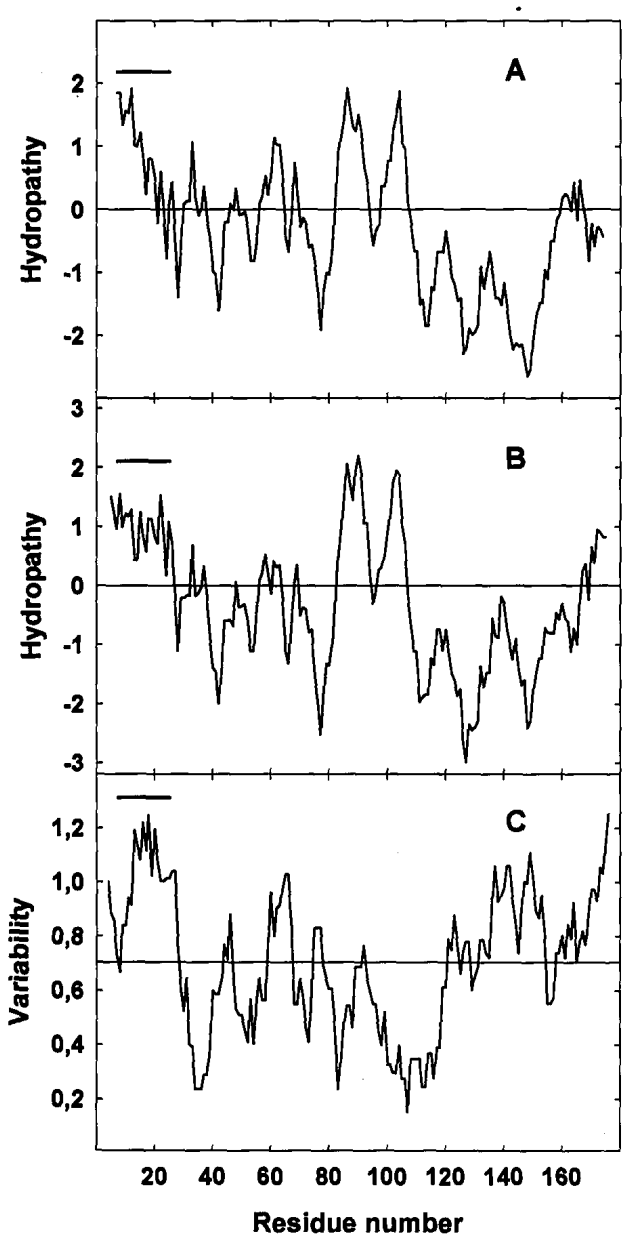

Fig. (2). Primary structure analysis of actinoporins. Hydropathy plots for (A) StnII and (B) Eqt II. Hydropathy values were calculated according to the scale developed in [83]. The horizontal bar corresponds to residues 5-28 in all three figure sections. (C) Variability plot was generated by computing the Shannon entropy [84] for each residue i as the average of individual entropies of residues i-3 to i+4. Only the sequences that are known in full appearing on Fig. (1) were analyzed. EqtII numbering was followed in all the plots for comparison purposes.

Early in the research on actinoporins, and based on conductivity measurements, it was suggested that oligomerization of the toxin might be needed for the establishment of the pore [26]. Kinetic analyses of vesicle permeabilization and chemical cross-linking experiments further supported the existence of oligomeric pores, though the calculation about the exact number of monomers involved varied between

three and four [27-30]. Within this same idea, StnII was shown to be a monomer-tetramer associating protein in solution [47]. On the contrary, no multimeric forms in solution have been detected for EqtII [44].

Thus, the specific mechanism of pore formation by actinoporins seems to match the general one described above for PFTs, with binding of the monomer to the membrane, oligomerization, and subsequent pore formation [29,48]. However, the regions of the protein that are involved in pore formation have only been envisaged yet mostly due to the successful structural studies developed in the last seven years. The next epigraphs deal with the description of the high-resolution structures of the water soluble state of EqtII and StnII, together with low-resolution models of actinoporins bound to lipids.

The Water Soluble Structure

So far, the only two water soluble structures of actinoporins known are those of EqtII and StnII. In the case of EqtII, both X-ray crystallography [49] and NMR [44] approaches have been employed, whereas for StnII only the former has been accomplished [50]. As it can be seen in Fig. (**3A**) (centre), both actinoporins share almost the same global fold. Both structures superimpose with an RMS deviation of 0.6 Å [50]. Two different views of the structures of both EqtII and StnII appear in Fig. (**3A**) (left and right, respectively). Both are built on a basis of a β-sandwich fold composed of twelve (for EqtII) or ten (for StnII) β-strands. This β-sandwich is flanked by two α-helices (Fig. (**3B**)) that interact with it through hydrophobic interactions, van der Waals contacts, and salt bridges. The analysis of actinoporins water soluble structure revealed the existence of an exposed cluster of aromatic amino acids (Phe106, Trp110, Tyr111, Trp114, Tyr131, Tyr135 and Tyr136 for StnII) that could be involved in the interaction with membranes. Accordingly, two residues of this cluster in EqtII, Trp112 and Trp116, have been shown to participate in membrane binding [48].

The structure of a StnII:phosphocholine (POC) complex was also solved by X-ray crystallography [50]. This led to the characterization of a POC binding site in StnII, which is partly hydrophobic (side chains of Val85 and Pro105, and aromatic rings of Tyr111 and Tyr135) and partly hydrophilic (side chains of Ser52 and Ser103 and hydroxyl groups of Tyr131, Tyr135 and Tyr136). The positive charge of choline moiety is stabilized by cation-π interactions with the aromatic rings of Tyr111 and Tyr135, whereas the phosphate group interacts with the phenolic hydroxyl groups of Tyr111 and Tyr136, and probably is further stabilized by the cationic side chain of Arg51. The structural changes associated with POC binding are very small (RMS deviation of 0.3 Å). However, some backbone modifications in the loops connecting strands β6 and β7 are detected. Remarkably, it is in this loop where the most significant differences between the NMR and X-ray derived structures of EqtII have been found [44]. Thus, the POC-binding site seems to participate in the initial recognition of the membrane. In agreement with this idea, POC is the polar head group of the lipids sensitive to actinoporins activity [30]. However, POC and StnII bind with a very low association constant in solution (unpublished observation), suggesting that this recognition must be significantly improved when established within the membrane environment. Such a behaviour has recently been observed for staphylococcal α-toxin [51], for example.

Apart from the cluster of aromatic amino acids and the POC-binding site, a region rich in basic residues has been proposed to play a role in the initial steps of membrane recognition *via* interaction with charged regions of the lipid head groups [44]. Such a region in EqtII would be comprised of Arg120, Lys123, Arg152, Lys159, and Lys178, together with the contiguous sequence Lys125-Arg126-Arg127. A separate and smaller patch of positive side-chains is centered on Lys30 and Arg79.

In the previous section, it was shown that the hydropathy profile analysis of actinoporins sequences showed three regions of high hydrophobicity (Fig. (**2A** and **2B**)). Two of those regions are located in the β5 and β6 strands (Fig. (**3B**)), where they form part of the hydrophobic core of the structure, being Val85 (StnII numbering) the only hydrophobic residue of this region that is exposed to solvent. Val85 forms part of the POC-binding site, as stated above. The third region was the N-terminal region, which is also the larger segment that can adopt a different structure without disrupting the fold of the β-sandwich core [49]. Then, it seems reasonable to correlate this high hydrophobicity of the N-terminal region with an eventual ability to establish deep interactions with the membrane core in the last steps of pore formation.

Very recently, it has been shown that the three dimensional structure of actinoporins is also shared by a family of fungal fruit-body lectins that bind the cell-surface exposed Thomsen-Friedenreich antigen (T-antigen) [52,53], such as XCL from *Xerocomus chrysenteron,* though the sequence identity between them and actinoporins falls below 15%. Comparison of both families of proteins [52] immediately shows the absence in the lectins of the N-terminal region of actinoporins mentioned above, in good agreement with the lack of permeabilizing activity of XCL. In addition, XCL lacks the structural elements required for POC recognition. Instead, the corresponding region seems to be optimal for carbohydrate binding, which agrees with its biological activity. Interestingly, the equivalent amino acids of another lectin, ABL from *Agaricus bisporus* have been shown to participate in the binding of sugar moieties [53].

Finally, exhaustive sequence databases searches have also allowed the identification of a good deal of sequences showing a stretch of significant degree of similarity with actinoporins and belonging to organisms from three different metazoan and two plant phyla [54,55]. This conserved sequence corresponds to a domain equivalent to the C-terminal region of actinoporins (83-179 EqtII residues) and would include the POC binding site. These actinoporin-like sequences are hypothesized to be intracellular proteins with the capability to attach to lipid membranes and, together with actinoporins and fungal fruit-body lectins, would conform a new superfamily of cell membrane binding domains.

In summary, the determination of the water soluble structures of EqtII and StnII revealed the existence of potentially important regions for actinoporins' functionality. The cluster of aromatic amino acids, for example, including the POC-

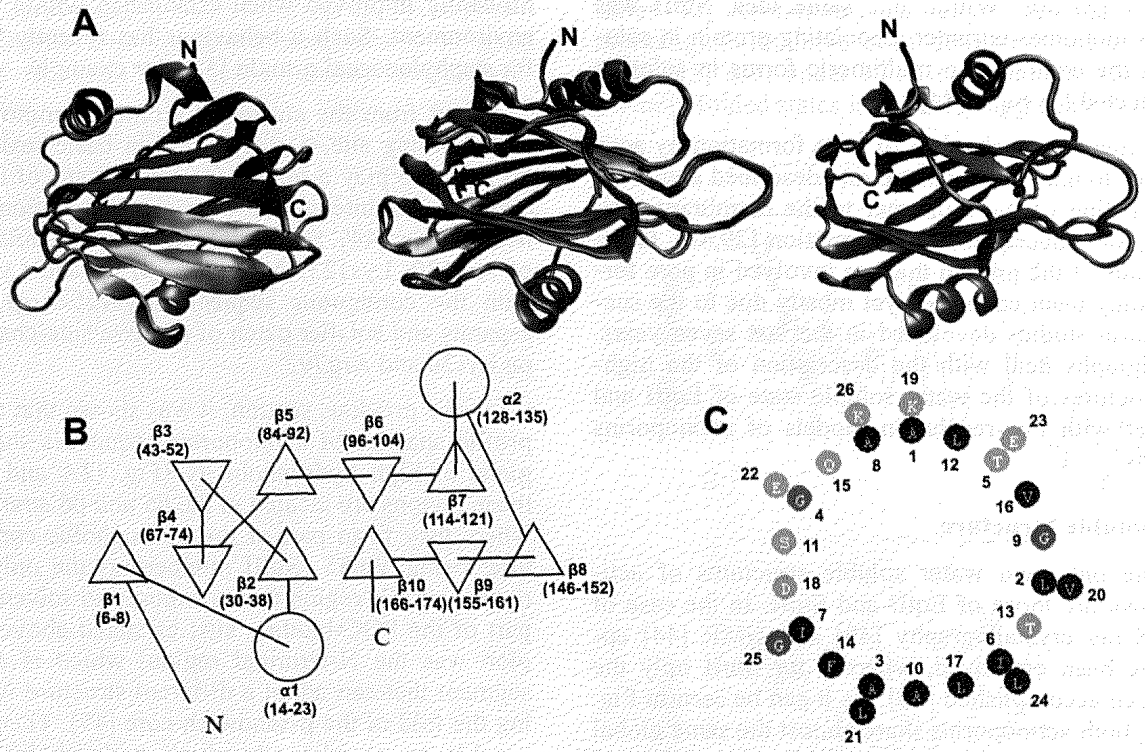

Fig. (3). Diagrams showing different aspects of the three-dimensional structure of the water soluble state of actinoporins. (A) EqtII (*left*, PDB code 1IAZ), StnII (*right*, PDB code 1GWY) and their superimposition (*centre*). The three diagrams show the proteins from three different points of view for a more generalized vision of the overall structure. N- and C-termini appear labelled. VMD [85] was employed to prepare the representations. (B) Diagram, constructed according to [86], showing the secondary structure distribution of StnII. Circles represent α-helical structure, whereas triangles correspond to β-strands. (C) Helical wheel analysis of StnII N-terminal region. This representation was constructed with Antheprot [87]. Residues shaded in black are hydrophobic.

binding site, together with a region rich in basic amino acids, seem to be optimal candidates for the initial establishment of the interaction with the membrane. Further conformational changes leading to pore formation would then occur within the N-terminus. Noticeably, actinoporins also show weak structural similarity with domain 4 of perfringolysin O, a Ch-dependent PFT from *Clostridium perfringens* [56]. This domain, which is responsible for the initial membrane recognition and binding of this much larger toxin, lacks the region equivalent to the N-terminal structure of actinoporins, and thus pore-formation requires the intervention of some of its other additional domains.

Non-Conductive Membrane Bound States

As explained above, PFT mechanisms of pore formation usually involve the establishment of a prepore intermediate [1]. Kinetic measurements using surface plasmon resonance (SPR) have shown that EqtII binding to membranes is a two-step process, thus confirming the existence of intermediates in their mechanism of pore formation [48,57]. The first step is driven at least in part by the aromatic cluster of amino acids, since steric shielding of this cluster and mutations of Trp112 and Trp116 to Phe significantly reduce the toxin-lipid interaction. In the second step, the N-terminal amphiphilic helix translocates to the lipid phase, as shown by the behaviour of a double cysteine mutant that has the N-terminus fixed to the protein core by a disulfide bond [48].

There has been some nice attempts to determine the structure of non-conductive lipid bound states of StnII by employing two-dimensional crystallization on lipid monolayers and electron microscopy (EM) approaches [50,58]. As this model system lacks one of the two hemilayers that conform a membrane, the formation of the conductive pore is hampered [59]. Interestingly, tetrameric ensembles were detected in all cases. Resolutions of 15 - 18 Å were achieved for the three-dimensional reconstruction of such tetrameric assemblies (Fig. (**4A**)), which opened the possibility of docking into them the high resolution water soluble structure [50], thus obtaining a model where the conformational changes associated with initial lipid binding can be envisaged. Whereas the β-sandwich fits perfectly into the EM map, suggesting that no conformational changes would occur in this region, some other regions of the protein fall out of the density envelope (Fig. (**4**)). These are the whole N-terminal region, the highly basic loop between strand β7 and helix α2 (amino acids from 121 to 127) and part of this helix (amino acids from 128 to 133). It is noteworthy to mention that some of the amino acids belonging to the POC-binding site are located in those regions of the protein. It was suggested that a simple pseudorigid movement of the N-terminal region could fill the EM density in between monomers [50]. With this conformational change, the α1 helix would adopt a nearly perpendicular orientation with respect to the membrane and would participate in monomer-monomer interactions with the C-terminal of the adjacent monomer. Such a conformational change would not expose hydrophobic amino acids belonging to the β-sandwich, with the exception of Leu70 (StnII numbering). Whether this EM-derived structure shown in Fig. (**4A**) is a real intermediate in the way to pore

formation is still far from being demonstrated. Two significant drawbacks can be pointed out. First, the distance between the closest residues of opposite-facing monomers is 5 nm, approximately 2.5 times larger than the diameter of the final pore (see next section). Second, the fact that the N-terminus mediates monomer-monomer contacts might hinder its insertion into the membrane during the last steps of pore formation. Both drawbacks may be overcome taking into account relatively large conformational changes, but there are still no conclusive experimental data supporting them in the literature.

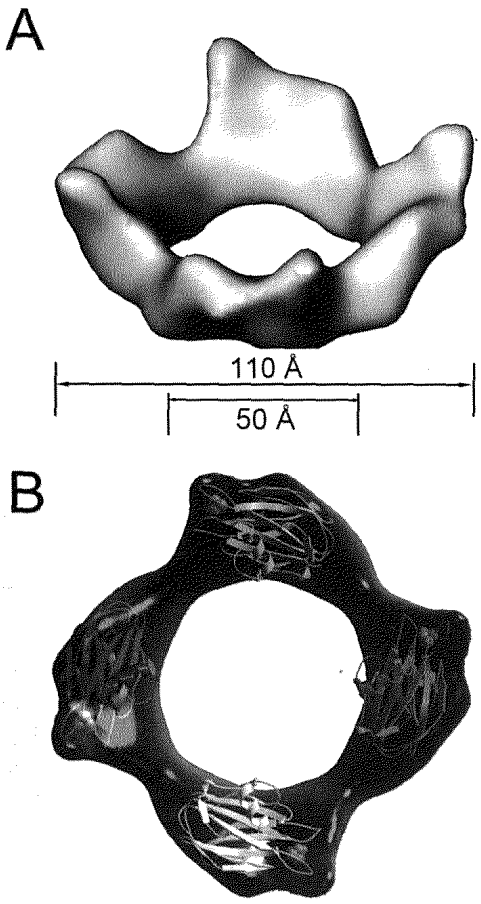

Fig. (4). Non-conductive lipid bound state of actinoporins. (A) View of the structure of StnII bound to DOPC monolayers determined by EM. (B) Docking of the high resolution water soluble structure of StnII into the low resolution electronic envelope of StnII bound to DOPC monolayers [50].

Some indirect approaches have also been employed for the study of the intermediates of pore formation. For instance, a detailed cysteine-scanning mutagenesis of the N-terminal region of EqtII detected that residues 10-28 are organized as an α-helix, which implies an extension nine amino acids longer than the water soluble state helix α1 [60]. Such an α-helix would have amphiphilic nature (Fig. (**3C**)). However, an orientation parallel to the membrane was predicted (the so-called M2 state), in agreement with another mutagenesis study [59]. This apparent contradiction with two-dimensional crystallization experiments was recently overcome by a complementary microscope analysis of new crystals of StnII [61]. Interestingly, the relative orientation of the actinoporin interacting with the membrane, as well as the absence of conformational changes in the β-sandwich that are predicted from these two-dimensional crystallization experiments, agree with ^{19}F NMR measurements performed in micelles and bicelles [62].

Thus, the study of the intermediates leading to pore formation again points out that the cluster of aromatic amino acids, the POC-binding site and the highly basic region are important for the initial membrane attachment and also confirms that conformational changes at the N-terminus of actinoporins must occur. In addition, a role for the N-terminal region in the oligomerization of the protein is suggested.

The Pore Structure

At the present time, there has been no report on a high resolution structure for the pore state of actinoporins. This situation probably only reflects the difficulties that are usually encountered when dealing with integral membrane proteins. The same lack of high-resolution structural data for this pore state is found within the whole family of PFTs, with the notable exception of staphylococcal α-toxin [1,63]. However, the actinoporins' pore state has been extensively studied by means of low-resolution methods, like FTIR and circular dichroism spectroscopies, as well as by more indirect approaches, like cysteine-scanning mutagenesis. Overall, they have provided enough structural data as to allow the construction of a putative pore model hypothesis.

The radius of the pore formed by actinoporins has been estimated to be around 1 nm by using osmotic protectants of different size [27,28,30,64] and by conductance measurements in planar bilayers [27]. For StnI, the pore size is independent of toxin concentration and similar in natural and model membranes [64]. This may imply that pores have a fixed common predominant structure. However, EqtII pores have a broad conductance distribution [27,65], suggesting that they may adopt slightly different conformations while keeping constant their size.

The use of cysteine-scanning mutagenesis has been of great importance for the determination of actinoporins topology in the pore state. This methodology is really suitable for actinoporins as the wild-type proteins lack cysteine residues (Fig. (**1**)). A low-resolution study showed that two regions of EqtII become embedded in the membrane, again the cluster of aromatic residues and the N-terminal helix [66]. The former is involved in the initial recognition of the membrane and therefore keeps interacting with the lipids after formation of the pore, whereas the latter seems to insert in the membrane during the last steps of pore formation.

When this cysteine-scanning mutagenesis approach was applied in deep detail to the study of the N-terminus of EqtII, it showed that residues 10-28 are organized as an α-helix in the pore structure [60]. Considering that this N-terminus is transferred across planar lipid membranes [65], it seems reasonable to postulate that the N-terminal helical extension also affects to the first amino acids, a possibility that was not investigated in that study [60]. As shown in Fig. (**3C**), such an extended helix would be of amphipathic nature. In agreement with this interpretation, truncation of the first ten residues of EqtII causes a 3-fold decrease of its hemolytic activity [67] and, if only the first five residues are the ones de-

leted, the mutant retains 83% of its activity. An inspection on the properties of this second mutant also shows that lower conductance pores are formed [65]. Such pores are not as stable as the wild type ones, suggesting again the involvement of the first five residues of EqtII in the correct establishment of the pore. Interestingly, a 32-residue peptide having the N-terminal sequence of EqtII has been shown to adopt α-helical conformation from residues 6 to 28 in dodecylphosphocholine micelles [68]. Similarly, the equivalent peptide for StnII also adopts α-helical conformation in the membrane environment mimicking agent trifluoroethanol [69]. Accordingly, FTIR [70,71] and circular dichroism [72] measurements have detected increments in the α-helical content of actinoporins upon lipid binding that are compatible with the extension of the N-terminal helix. A slight increase in β-structure has also been detected by FTIR, though no clear interpretation can be currently given [71], as neither the β-sandwich nor the C-terminal α-helix seem to undergo large conformational rearrangements during pore formation [73].

The angle between the N-terminal helix and the membrane normal has been estimated by infrared linear dichroism to be about 31° [71], in good agreement with the model proposed by Malovrh *et al.* [60] based on ion current data of methanethiosulfonate (MTS)-modified Cys mutants. In such a study [71], the orientation of the β-sandwich was also estimated, being 47° the value found for the average orientation of the carbonyl bond with respect to the normal of the membrane. Finally, it is also known from $^{1}H/^{2}H$ exchange [71] and fluorescence [48] experiments that actinoporins do not insert deeply in the membrane except for its N-terminus. Taking into account all the above data, a model of the actinoporin protomer within the pore structure has been proposed (Fig. (**5**)).

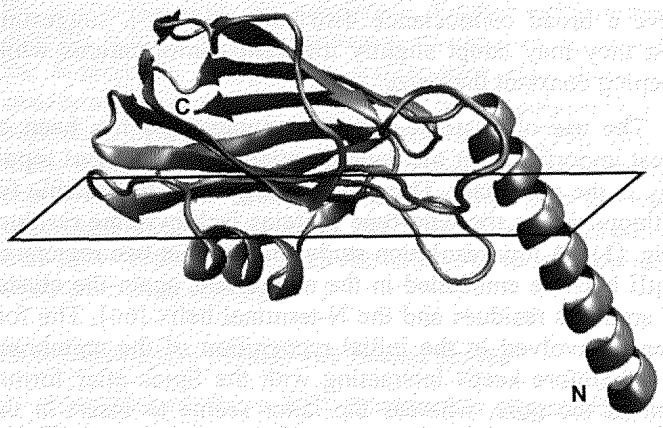

Fig. (5). Hypothetical conformation of actinoporins within their pore state. Diagram was generated with VMD [85] and PyMol (DeLano Scientific, San Francisco, CA) from the coordinates of the water soluble structure of StnII (PDB code 1GWY). Membrane plane is indicated and N- and C-termini appear labelled.

The specific positioning of the N-terminal α-helix in the pore has been studied by means of conductance measurements in planar lipid bilayers. Residues 1, 3, 10, 14, 17, 21, 24 and 28 of EqtII (1, 8, 12, 15, 19, 22 and 26 in StnII) have been proved to face the pore lumen, in agreement with their general hydrophylic character (Fig. (**1**)) [60,65]. The most hydrophobic residue within them is Leu14, a position that is conserved in all actinoporins' sequences (Fig. (**1**)). It was proposed that this residue is positioned at the very border of the hydrophobic face [60]. Those studies agree with the extension of the N-terminal amphipathic helix (Fig. (**3C**)) and with the existence of titrable negative charge groups (Asp3, Asp10, Asp17 and Glu 24) in that pore lumen, where they would modulate ion permeability and selectivity [26].

It is difficult to conceive a tetrameric pore with a resulting radius of 1 nm, as it seems to be the case, if it is formed by only four monomers as the one shown in Fig. (**5**). Then, at least one of the following two options must occur: Either other regions of the monomer participate in the pore walls or lipids form part of the conductive channel giving rise to a toroidal pore, or both. The second option is the one being currently considered to be more feasible. For instance, actinoporin-induced orientation change of lipid acyl chains has been detected both by FTIR [38,70,71] and by ^{31}P NMR [38]. The formation of a toroidal pore would also explain why the inclusion of lipids that induce negative curvature in the bilayer helps in the formation of the pore [37]. Further evidence comes from EPR experiments, which indicate a lack of lipid-protein interactions in regions close to the methyl terminal group of the lipid acyl chains [39].

To summarize, all evidence to date suggests that the N-terminal region of actinoporins is of vital importance for the formation of the functional pore, which might be in apparent contradiction with the fact that the most variable region in actinoporins is precisely the N-terminus (Fig. (**2C**)). However, this variability is located mainly at the hydrophilic residues, whereas a higher degree of conservation is observed for hydrophobic ones (Fig. (**1**)). Thus, it seems feasible that distinct actinoporins may form pores with slightly different conductivity properties, resulting in differences in their toxicity. If that is the case, the commonly observed fact that one specific anemone produces several isoforms of actinoporins might be explained in terms of a broadening strategy of its venomous condition. In this regard, it has been shown that the change of a single Glu by Gln at the N-terminal at position 16 of StnI produces a variant with a behaviour more similar to StnII, which in fact possesses Gln at the equivalent position [74].

MUTAGENESIS STUDIES

The production of functionally-impaired mutant variants of proteins is a powerful tool to assign specific functions to specific residues, or regions, of a protein. This strategy has only been used occasionally for actinoporins. In fact, the main objective of the majority of those studies has not been the identification of the most relevant residues for actinoporins' activity, but the heterologous production of the proteins in recombinant form (see for example [22]) or the determination of their topology when embedded into membranes [60,66]. However, the production of site-directed mutants should be also useful to understand the mechanism of pore formation by actinoporins. In Table **3**, all the mutants with reduced hemolytic activity that have been so far purified are presented, with the exception of mutants affecting multiple residues, which have been intentionally dismissed as the effects observed with them are difficult to assign to individual residues. The spatial location of these residues

throughout the EqtII three-dimensional structure is also shown in Fig. (**6**). Even when these mutants were produced in random mutagenesis experiments [75], or even when they were not specifically designed for studying the mechanism of pore-formation, they still sustain the importance of all the "actinoporins" key regions pointed out in the sections above. For instance, mutation of residues belonging to the cluster of aromatic amino acids and/or the POC-binding site (W112F and W116F mutants in EqtII and F106L and Y111N in StnII) impairs functionality. One of them is even a mutation (EqtII R126C) that affects to the basic region presumably involved in establishing contact with the membrane. However, the majority of the mutations affecting the hemolytic activity of the proteins are located at their N-terminus (A12C, S13C, K20C, V22W, L26C, G27C, N28C in EqtII and K19E in StnII), in agreement with the vital role of this region. Within this same idea, both the addition and the deletion of amino acids at the N-terminus render less active variants too (Table **3**). Both situations may be explained by a defective pore formation due to the smaller size of the α-helix, when a deletion has taken place, or to a steric impediment that would account for the reduction in activity when the addition of extra amino acids is what has occurred. Accordingly, the longer the tag, the lesser the activity (Table **3**).

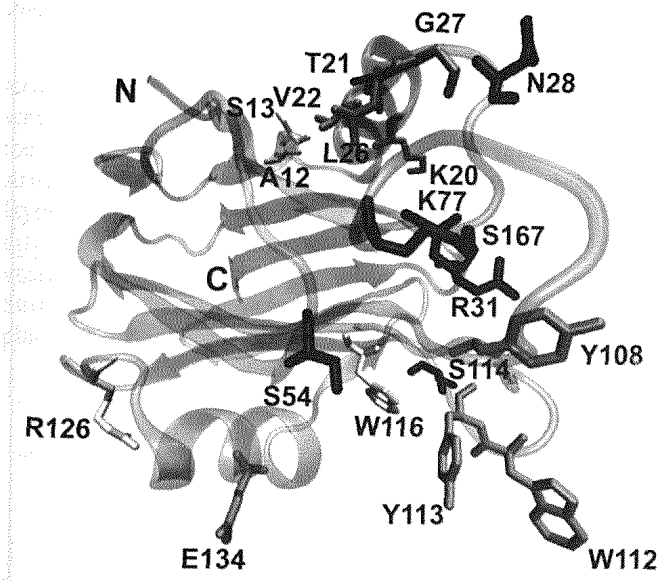

Fig. (6). Key residues for the functionality of actinoporins. Single-point mutations presented in Table **3** are represented in EqtII structure (PDB code 1IAZ). Residues are drawn according to the remaining activity of the mutant: light traces (50 – 90% activity retention), intermediate traces (15 – 50%) and dark traces (less than 15%). N- and C-termini appear labelled. VMD [85] was used to build the figure.

There are also some mutations which are located in unexpected regions of the protein. All of them, with the only exception of E134C in EqtII, are situated at the loops between the cluster of aromatic residues and the N-terminus, e.g. α1-β2, β3-β4, β4-β5, β6-β7 loops in StnII. Very interestingly, these mutations seem to be highly pernicious for the hemolytic activity, as none of them retains more than 15% of the wild-type values (Fig. (**6**) and Table **3**). Remarkably, precisely loop β6-β7 in StnII has been shown to suffer some backbone modifications upon POC binding [50]. All these data suggest that the interfacial region between the putative phospholipid-binding site and the N-terminus may play a crucial role in the functionality of actinoporins, with the probable function of transducing the signal of lipid binding to the N-terminal region, which then would proceed to the last steps of pore formation. However, it cannot be ruled out another role for that region during the oligomerization step.

A distinction of the mutants appearing in Table **3** can be made if their ability to bind lipids is also considered. Then, mutants affecting the cluster of aromatic amino acids or the basic region (EqtII W112F, W116F and R126C) are less active because they are worse at interacting with membranes, for example. A very similar situation is found for EqtII K20C and L26C, which suggests that mutations at the N-terminus may, at least in some cases, impair the initial steps of pore formation. In fact, lysine residues are important to establish superficial interactions with membranes because of their ability to "snorkel" the bilayer [76]. With respect to Leu26, this residue was shown not to be linked with pore lumen [60], what suggests that it is involved in the interaction with the hydrophobic core of the membrane. The fact that EqtII E134C also has a decreased ability to bind lipids suggests that acid residues may also be important for membrane recognition, perhaps by interaction with the positive-charged group of neutral phospholipids. On the other hand, the majority of mutants affecting the N-terminus present an equivalent ability to bind membranes than the wild-type sequences (Table **3**), which agrees with the idea that the main function of this region is not to participate in the initial attachment to the membrane but rather take part in the pore formation step. Remarkably, all the mutants affecting the loops connecting the N-terminus and the lipid-binding regions maintain their lipid-binding properties, also in agreement with their potential signal-transducing function. In particular, results obtained with EqtII R31C suggest that the cluster of basic residues centered on Lys 30 and Arg 79 may not be determinant for membrane recognition.

Very few of the above mutants have been studied more thoroughly. Notable exceptions are the His_6-tagged versions of EqtII and StnII, and EqtII Δ5, V22W, and K77C. Unexpectedly, while the tagged version of StnII forms larger pores than the wild-type protein [77], these pores have lower conductance values when His_6-EqtII is the protein studied. This lower conductance has been used indeed to explain the lower toxicity of this mutant [65]. The same study revealed that the N-terminus of His_6-EqtII is transferred across planar membranes and that the first five amino acids of EqtII stabilize the transmembrane pore. Regarding EqtII V22W, it was proposed that the newly introduced Trp would stabilize the mutant in a state prior to final pore formation due to its affinity for the lipid-water interface [59]. In such a state, the N-terminal helix would lie parallel to the plane of the membrane. Finally, EqtII K77C was also shown to form larger pores but these ones showed higher conductance than wild type EqtII. The activity of the mutant was recovered by chemical modification of the thiol group, provided that a positive charge was reintroduced [78]. These facts were interpreted in terms of a defective development of the oligomer needed in order to lead to pore formation.

Table 3. Mutant Variants of Actinoporins with Reduced Activity. Lipid binding and permeabilizing activities of the mutants are compared with those of the wild-type proteins. Symbols are given according to the percent of retained binding or permeabilizing activity with respect to the wild-type sequences. (=) > 90%, (-) 50-90 %, (--) 15-50 %, (---) < 15%.

Protein	Mutant	Binding	Permeabilizing activity	Reference
EqtII	His_6-tagged (N-terminus)	n.d.	--	[65][a]
	TolA-tagged (N-terminus)	=	---	[48][a,d]
	Δ1-5*	n.d.	-	[67][a]
	Δ1-10**	n.d.	--	
	A12C	=	--	[60][a,d]
	S13C	=	-	[66][b,e]
	K20C	-	--	
	V22W	=	--	[59][a,e]
	L26C	--	--	[60][a,d]
	G27C	=	--	
	N28C	n.d.	---	
	R31C	=	---	[66][b,e]
	S54C	=	---	
	K77C	-	---	
	W112F	--	--	[48][c,f]
	S114C	=	---	[66][b,e]
	W116F	-	-	[48][c,f]
	R126C	-	-	[66][b,e]
	E134C	-	--	
	S167C	=	---	
StnII	His_6-tagged (N-terminus)	=	--	[77][b,f]
	K19E	n.d.	---	[75][a]
	F106L	n.d.	--	
	Y111N	n.d.	--	
StnI	His_6-tagged (N-terminus)	n.d.	---	[22][b]
HmgIII	Gly-Ser Tag (N-terminus)	n.d.	--	[96][a]
SrcI	Thioredoxin-tagged (N-terminus)	n.d.	---	[15][a]

* The first five residues were deleted. ** The first ten residues were deleted.
[a] Hemolytic activity was determined. [b] Hemolytic and liposome permeabilizing activities were determined. [c] Liposome permeabilizing activity was determined. [d] Binding to red blood cells was determined. [e] Binding to liposomes was determined. [f] Binding to supported planar membranes was determined by SPR.
n.d. Not determined.

In short, the data summarized in this section clearly show that site-directed mutagenesis offers valuable possibilities of gaining insight into the mechanism of pore formation by actinoporins at the molecular level. It is therefore predictable that in the near future, new mutagenesis studies will be presented.

THE CURRENT VIEW OF THE MECHANISM OF PORE FORMATION

Even though the extensive research performed in the last years has yielded a good amount of information regarding actinoporins' pore formation mechanism, there are still many aspects that remain obscure and this mechanism is still far

from being understood in full. Therefore, this section aims to summarize the current knowledge as a putative model that would try to explain at the molecular level the mechanism of pore formation by actinoporins.

The compilation of all the data presented in the sections above leads to a model of pore formation that comprises at least four steps (Fig. (7)). In the first one, actinoporins would bind the membrane via the cluster of aromatic amino acids, the POC-binding site, and the basic regions, though additional interactions involving acidic residues may be occurring (see results described above for mutant EqtII E134C for example). The structure of this bound species, M_o-state, would be essentially the same as that of the water soluble state (Fig. (**3A**)). SM may enhance this initial interaction if present in the target membrane [35], in agreement with its ability to inactivate actinoporins by preincubation. The absence of permeabilization of pure SM liposomes may reflect that the M_o-state might not proceed to the latter steps of pore formation in that case. The existence of this M_o-state has been proven by EqtII V8C/K69C double cysteine mutant, which has the N-terminus fixed to the protein core by a disulfide bond [48]. When oxidized, this mutant is inactive, though it binds lipids normally. In reducing conditions, EqtII V8C/K69C is able to dissociate the N-terminal helix and therefore recovers its activity.

In the second stage, a pseudorigid movement of the N-terminus along the loop between helix α1 and strand β2 would separate the helix from the β-sandwich giving rise to the M_1-state. Such a state is compatible with the EM-derived structure obtained from two-dimensional crystals on monolayers (Fig. (**4**)) [50], although the possible coincidence of this structure with that of the M_1-state has to be further investigated. In that structure, tetrameric lipid-bound forms of StnII were found, which suggests that oligomerization takes place in this state. The N-terminal region would participate in the establishment of monomer-monomer interactions with the C-terminal region of the adjacent monomer. In the transition from M_o to M_1, the region involving α1-β2, β3-β4, β4-β5 and β6-β7 loops (StnII numbering) would play the role of functionally connecting the lipid-binding regions to the N-terminus of the protein (Fig. (**6**)).

The third step would be the extension of the N-terminal helix that would lie parallel to the membrane, resulting in the M_2-state. Again, the existence of this state has been assessed by EM [61]. In addition, EqtII V22W mutant has been proposed to be less active because of the stabilization of the M_2-state as a consequence of the high affinity of tryptophan for the lipid-water interface [59].

Finally, the extended N-terminal helix would penetrate into the membrane to build the pore. Each monomer would adopt a structure similar to the one presented in Fig. (**5**). The distinct size of the arrows in Fig. (**7**) indicates that once the pore is formed, it is extremely difficult to go back. In fact, it is commonly accepted that the process of pore formation is irreversible [26], though equilibrium constants have been measured [48]. Results from EqtII Δ5 mutant show that the five first amino acids would be important for the stabilization of the pore structure [65], which would include lipids in the conductive channel. Then, a toroidal lipid pore would be formed. In this way, the inclusion of lipids that favour negative curvature in the membrane would facilitate the transition from M_2-state to the pore structure.

PERSPECTIVES

The large amount of experimental available data sustain the above putative model for pore formation. However, there are still some aspects far from being well-characterized. For instance, to date, the kinetic and/or thermodynamic importance of the proposed intermediates has not been studied in detail. Oligomerization is probably the most controversial

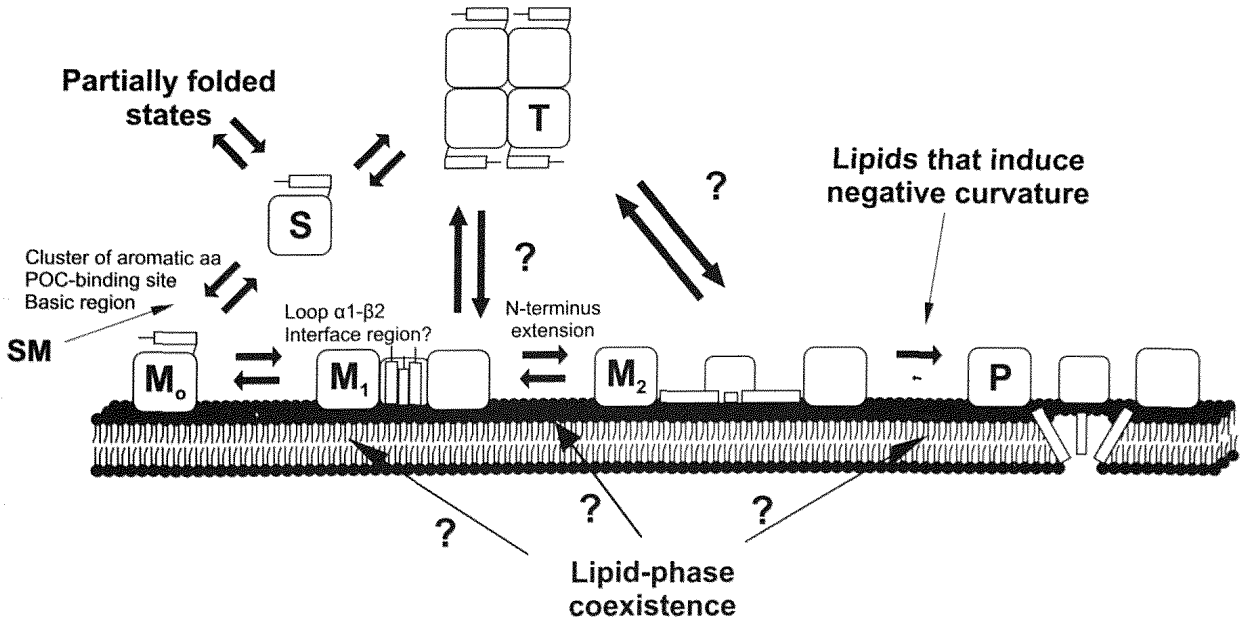

Fig. (7). Putative model of pore formation by actinoporins. Monomers are drawn as a square (representing the β-sandwich and the helix α2) and a rectangular appendix (representing the N-terminal α-helix). S: water soluble monomer. T: water soluble tetramer. M_o: monomer bound to the membrane. M_1 and M_2: tetrameric non-conductive lipid-bound forms. P: tetrameric pore. For the sake of simplicity, only three monomers are represented in M_1, M_2 and P.

step. Though it seems clear that four monomers associate to form the pore, there is no knowledge on when oligomerization takes place exactly. It has been suggested that interaction with lipid membranes would promote an increased protein concentration at the bilayer surface favouring oligomerization [47]. Then, oligomerization would occur concomitantly with the dissociation of the helix α1 from the β-sandwich (M_0 - M_1 step, Fig. (7)). However, there is no experimental data yet to discard the possibility of oligomerization happening simultaneously with membrane binding (S - M_0 step, Fig. (7)). Even the possibility that water soluble tetramers (T, Fig. (7)) bind to membrane cannot be ruled out, though there is some reasonable evidence that membrane binding by actinoporins precedes oligomerization [29,79]. In addition, some authors postulate that the M_2-state is monomeric [60]. Currently, nothing is known about the possible similarity of the tetrameric structures in solution to those within membranes. It also must be kept in mind that the presence of multimeric forms in solution has only been detected for StnII [47], but neither for EqtII [44] nor any other actinoporin. Then, such ability to tetramerize in solution may not be a common feature of actinoporins.

Another question to be answered is why phase coexistence in the target membrane promotes pore formation by actinoporins. It seems clear that phase coexistence has nothing to do with initial binding, as demonstrated by the tight binding of actinoporins to pure SM membranes, which do not support phase coexistence. One possible answer is that actinoporins may have some affinity for the interfaces between coexisting lipid phases [34]. Then, monomers would be confined in a one-dimensional space resulting in a very efficient mechanism of concentration. Then, subsequent oligomerization leading to pore formation would be easier. Additionally, the interfaces between coexisting phases are prone to lipid packing defects [80]. Actinoporins may therefore take advantage of such defects to proceed to the last steps of pore formation.

To solve the above uncertainties, the characterization of mutants affecting different stages of pore formation will be of the highest interest. In addition, the study of the thermodynamics of pore formation may offer new valuable information. Finally, there is a need to obtain direct high-resolution data of the pore structure. In this regard, the isolation of a stable soluble pore assembly would be of great help in order to understand the precise role of lipids in the pore, or what is the exact length of the N-terminal helix in the pore. Such structural studies may be hindered by the possibility that the pore structure is not a unique entity, as suggested by the broad conductance distribution of actinoporins pores. Undoubtedly, all of those experimental approaches will be undertaken in the future to better understand the mechanism of pore formation by actinoporins.

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ABBREVIATIONS USED

Ch = Cholesterol

EM = Electron microscopy

EPR = Electron paramagnetic resonance

Eqt = Equinatoxin

FTIR = Fourier-transform infrared spectroscopy

MTS = Methanethiosulfonate

MW = Molecular weight

PC = Phosphatydilcholine

pI = Isoelectrical point

POC = Phosphocholine

PFTs = Pore-forming toxins

SM = Sphingomyelin

SPR = Surface plasmon resonance

Stn = Sticholysin.

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